

Diagnosis of and Screening for Cytomegalovirus Infection in Pregnant Women

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No single diagnostic test for cytomegalovirus (CMV) infection is currently available for pregnant women at all stages of gestation. Improved accuracy in estimating the timing of primary infections can be used to identify women at higher risk of giving birth to congenitally infected infants. A diagnostic algorithm utilizing immunoglobulin G (IgG), IgM, and IgG avidity was used to prospectively screen serum from 600 pregnant women enrolled from two groups: ≤ 20 weeks gestation ($n = 396$) or > 20 weeks gestation ($n = 204$). PCR testing of urine and/or blood was performed on all seropositive women ($n = 341$). The majority (56.8%) of women were CMV IgG seropositive, with 5.5% being also CMV IgM positive. In the IgM-positive women, 1.2% had a low-avidity IgG, indicating a primary CMV infection and a high risk of intrauterine transmission. Two infants with asymptomatic CMV infection were born of mothers who had seroconverted in the second trimester of pregnancy. Baseline, age-stratified CMV serostatus was established from 1,018 blood donors. Baseline seropositivity from a blood donor population increased with age from 34.9% seroprevalence at less than 20 years of age to 72% seroprevalence at 50 years of age. Women at high risk of intrauterine transmission of CMV were identified at all stages of gestation. Women infected with CMV during late gestation may be more likely to transmit the virus, so failure to detect seroconversions in late gestation may result in failure to detect infected neonates.

Human cytomegalovirus (CMV) is the most common cause of congenital malformation resulting from viral intrauterine infection in developed countries (12, 21, 48). Primary CMV infection occurs in 0.15 to 2.0% of all pregnancies and may be transmitted to the fetus in up to 40% of cases (48). Up to 15% of intrauterine CMV infections result in symptomatic congenital disease at birth, and 10 to 15% of those born with asymptomatic congenital CMV will develop significant clinical sequelae in infancy (7, 10, 18). In utero transmission of CMV can occur during primary maternal infection, reactivation, or reinfection of seropositive mothers. Most concern centers on primary maternal infection, due to the potential for significant fetal damage when the infection is acquired and transmitted during the first trimester (30, 48). Perinatal infections can result through virus transmission from many parts of the birth canal (39); however, the majority of these infections are asymptomatic (43).

The usefulness of prenatal testing for CMV has been questioned due to the absence of clearly effective intervention (1, 27) and to evidence for severe congenital malformation resulting from viral reactivation (6, 8, 20). Continuing advancements in technology, however, mean reliable and inexpensive serologic tests are available, prenatal diagnostic procedures with acceptable negative predictive values (NPV) can be per-

formed, and trials of neonatal antiviral treatments are ongoing (25, 34, 37, 50, 52). Proposed diagnostic algorithms have focused on first-trimester screening, since the time of infection can be accurately obtained in the absence of seroconversion data, and the clinical sequelae of congenital CMV is usually more severe if transmission occurs early in gestation (30, 48). A high positive predictive value (PPV) and NPV for clinical disease have been determined for quantitative PCR testing of amniotic fluid (26); however, there is an increased risk of a false-negative result if fewer than 7 weeks have elapsed between the onset of maternal infection and the time of amniocentesis (5, 36). In addition, amniotic fluid testing prior to 21 weeks gestation only has a 30 to 45% sensitivity rate, while testing after 21 weeks gestation increases the sensitivity to 74% (13, 35). Such time constraints are important if termination is considered as a management option, since the gestational age of a viable fetus is currently considered to be ca. 24 weeks (9). Amniocentesis also increases the risk of spontaneous abortion (2, 49), which in some cases may be greater than the risk of intrauterine CMV transmission.

Many parents desire antenatal diagnosis of intrauterine infection so that they are informed of the possible outcomes for their child, as opposed to antenatal testing for selective termination (43). There is therefore a need for a low-risk, noninvasive diagnostic test. Laboratory methods are required to diagnose acute CMV infections since most present nonspecific symptoms. Women are not routinely screened for CMV prior to conception so CMV seroconversion is infrequently documented, making diagnosis of primary CMV infections difficult.

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The presence of CMV-specific immunoglobulin M (IgM) may not be indicative of primary infection, since it is also produced during reactivation and reinfection (41). IgG antigen avidity has been used to clarify primary or nonprimary infections by measuring the binding affinity of IgG antibodies. IgG of low avidity are produced at the onset of infections, and subsequent maturation of the antibody increases its avidity over time. The use of IgG testing was pioneered in a large scale study on toxoplasmosis (31) and has more recently been shown to be useful for distinguishing primary and nonprimary CMV infections (3, 24, 33).

Furthermore, since the risk of CMV intrauterine transmission increases with advancing gestation, there is a need for diagnostic tests that can be used at all stages of gestation. In developing an appropriate diagnostic algorithm, test sensitivity, specificity, PPV, and NPV need to be estimated, utilizing known population prevalence. We outline here a noninvasive, diagnostic algorithm for congenital CMV detection at all stages during pregnancy based on initial serological screening with CMV IgG, IgM, and IgG avidity. The diagnosis of asymptomatic neonates is emphasized, since half of the children who suffer from CMV sequelae are asymptomatic at birth (10, 18). Also, knowledge of the risk of conditions such as sensorineural hearing loss in asymptomatic neonates, can allow close monitoring, early diagnosis, and early intervention, whereas failure to detect and follow up asymptomatic neonates may have serious consequences for the development of the child (51).

MATERIALS AND METHODS

Patients. Two cohorts were studied. The first cohort consisted of 1,018 de-identified blood donors, who were tested for CMV IgG serostatus between 30 October 2003 and 10 November 2003 as part of routine screening. The second cohort consisted of 600 pregnant women, prospectively screened for CMV by serology from 7 October 2002 to 1 June 2004. The women were presenting for routine antenatal care at a tertiary referral women's hospital (the Royal Hospital for Women), which has ca. 3,500 confinements each year. All subjects gave written consent under the South Eastern Area Health Services ethics approval 02/085 and the University of New South Wales ethics approval 03110.

Specimens. Whole blood and urine samples were collected from all women in the second cohort. Whole blood samples were centrifuged at $2,000 \times g$ for 20 min, and the buffy coat was removed and stored at -20°C . DNA was extracted from urine either on the day of collection or after storage at 4°C for a maximum of 24 h. DNA was extracted from urine and buffy coat by column purification using QIAamp DNA minikits (QIAGEN, GmbH) according to the manufacturer's protocol.

PCR was used to detect the excretion of CMV in the urine samples obtained. Viral isolation was conducted on all CMV-positive urine samples. Virus isolation was performed by using a standard shell-vial assay (22) on MRC5 cells, followed by detection using a Light Diagnostics kit (Chemicon). The direct immunofluorescence technique identifies the immediate-early antigen of human CMV. Women who were CMV IgM positive were also tested by PCR for CMV DNA in their blood.

Serology. CMV IgG and IgM were detected in patient serum by using a commercial microparticle enzyme immunoassay (Abbott AxSYM; Abbott Laboratories). An IgG avidity assay (CMV IgG avidity EIA; Radim, Rome, Italy) was used to distinguish between primary and recurrent CMV infections (32). A second commercially available CMV-IgM enzyme immunoassay (Eti-Cytok IgM; Sorin Biomedica, Vercelli, Italy) was also used on all sera. The procedures and interpretation of results were performed according to the manufacturer's instructions except that specimens with an avidity index of $\geq 35\%$ were considered high avidity.

PCR. A solution phase nested PCR was carried out on the DNA extracted from all samples with primers specific to the major immediate-early (MIE) section of the CMV genome. The first round amplified a 416-bp region using the designed primers MIE1517 (sense; 5'-AAGGTTCGAGTGGACATGGT-3') and MIE1932 (antisense; 5'-CAGCCATTGGTGGTCTTAGG-3'). A combina-

tion of previously described primers were used for the inner round to amplify a 249-bp product: MIE1661 (sense; 5'-GAGCCTTCGAGGAGATGAA-3') and MIE1909 (antisense; 5'-GGCTGAGTTCTTGGTAAAGA-3') (28). The cycling conditions for both rounds consisted of denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 50 s. A final incubation at 72°C for 3 min ensured that all amplified products were complete. A DNA positive control amplification was carried out on all samples using a single-round PCR to the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene (14). The GAPDH PCR was performed concurrently with the MIE PCR under the same cycling conditions.

Statistical analysis. Normally distributed continuous variables were compared by using a two-sample Student *t* test. Cross-tabulation and chi-square (with Yates' continuity correction) or Fisher exact tests were used to examine the relationship between variables using a 95% confidence interval as a measure of association.

RESULTS

A baseline rate of CMV IgG seropositivity was obtained by screening 1,018 de-identified blood donors from the Australian Red Cross Blood Service (ARCBS) in Sydney (cohort 1). In subjects younger than 20 years of age, approximately one-third (34.9%) were CMV IgG seropositive. The CMV seropositivity rate steadily increased with age, reaching a plateau at the age of 50, with more than two-thirds (72.4%) of the population infected. There were approximately equal numbers of men (48.5%) and women (51.5%) within the population; however, there were significantly more ($P = 0.05$) CMV-seropositive women (54.4%) than men (45.6%).

The CMV seropositivity rate for the pregnant women cohort showed that, overall, 56.8% women were CMV IgG positive at pregnancy. The age range of the pregnant women was 19 to 47 years, with no significant difference seen between the mean age of seropositive and seronegative women. Compared to the ARCBS population, the CMV seropositivity rate in the pregnant women cohort was higher at less than 20 years of age (60% compared to 34.9%) and between the ages of 20 to 30 years (66.27% compared to 44.8%) but was similar after the age of 30 years (50% compared to 56.6%).

The diagnostic algorithm used for screening the prospective cohort of 600 pregnant women is shown in Fig. 1. The women were divided into two groups based upon gestation. Group A consisted of 396 patients who consented through the outpatient clinic at 20 weeks or less than 20 weeks of gestation (mean, 15 weeks). Group B consisted of 204 patients who consented at the time of glucose tolerance testing (GTT) for gestational diabetes at over 20 weeks gestation (mean, 28 weeks). All women were screened serologically for CMV IgG and IgM. Women with an equivocal serology result or an IgG-negative and IgM-positive result were screened 3 weeks later to confirm the serostatus of the patient. CMV IgG avidity was determined for all patient specimens that were CMV IgM and IgG positive. First-trimester bleeds from women in group B who were IgM positive were screened retrospectively. No statistical differences ($P > 0.05$) were seen in the average age, parity, or CMV serostatus of the two groups.

Of the 600 women tested, 259 (43.2%) had never been infected with CMV and 308 (51.3%) had a past CMV infection (Table 1). The remaining 33 (5.5%) women were CMV IgM positive, 25 (4.2%) of whom were from group A and 8 (1.3%) of whom were from group B. Of the IgM-positive women, five from group A and two from group B had low IgG avidity, a

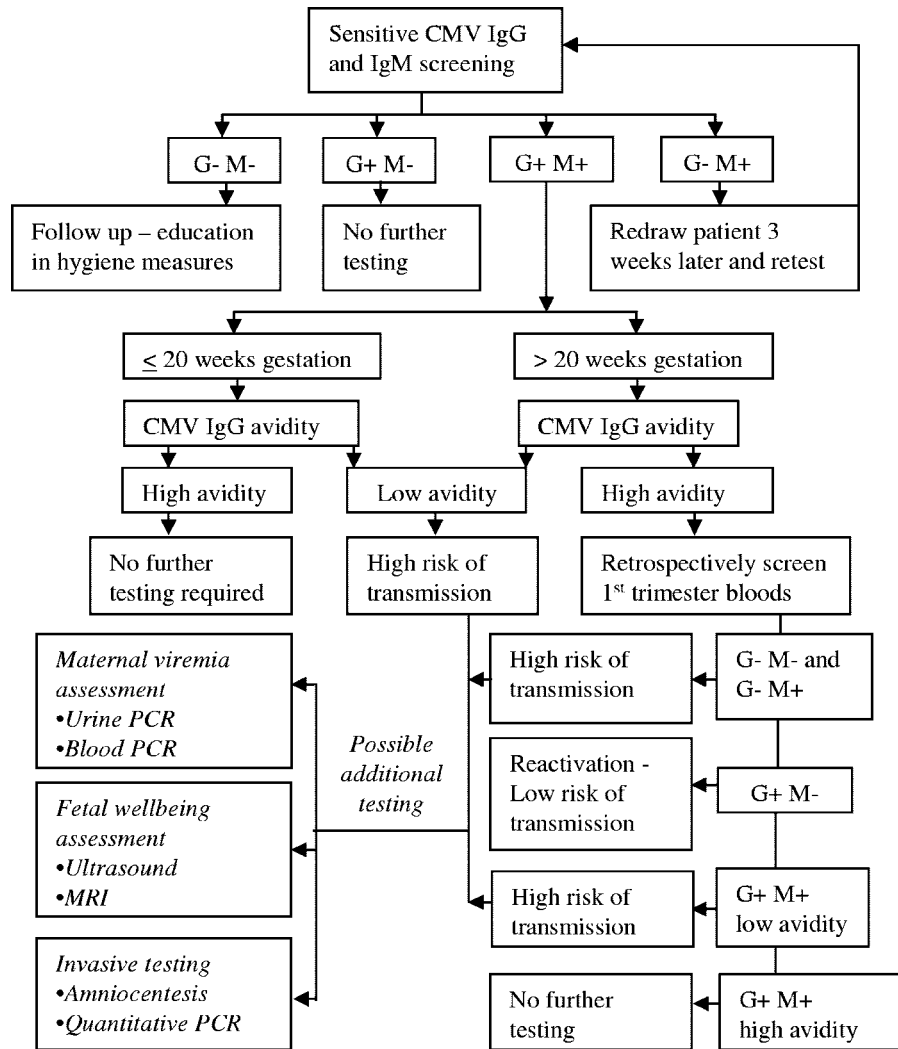


FIG. 1. Proposed diagnostic algorithm for CMV serology screening in pregnant women.

finding consistent with a primary CMV infection and a high risk of intrauterine transmission (Table 2). Retrospective testings of the first-trimester screens from the two women from group B showed that they were CMV seronegative in their first trimester, indicating seroconversions during the second trimester of pregnancy and a high risk of CMV intrauterine transmission.

A second commercially available CMV-IgM enzyme immu-

noassay (Eti-Cytok IgM) was used on all sera, and the results were compared. The CMV IgM reactivity rates for the AxSYM/IMx and the Eti-Cytok IgM levels were 5.7 and 1.8%, respectively. In the seven IgM-positive cases with low-avidity IgG, the Eti-Cytok IgM test failed to detect two cases (Table 2). Another five discordant specimens were CMV IgM negative in the AxSYM/IMx test but positive in the Eti-Cytok IgM test. All of these specimens contained high-avidity IgG.

PCR was used to detect excretion of CMV in urine from mothers and infants. Of the seropositive women, 1.8% (6 of 341) were shown to be shedding CMV at the time of testing with 4 of the women being IgM positive, 3 of whom had a low-avidity IgG level. CMV IgM positive women were also tested for the presence of CMV DNA in their blood. Four of the CMV IgM-positive women were CMV DNA viremic; all of these woman had low-avidity IgG (Table 2).

All IgM-positive mothers and an equal number of control patients were monitored through an infectious disease clinic until birth. Mothers in the control groups were CMV IgG negative and IgM negative or CMV IgG positive and IgM negative. One IgM-positive woman with a low-avidity IgG re-

TABLE 1. Serological data collected prospectively between 7 October 2002 and 1 June 2004 for 600 pregnant women^a

CMV immunoglobulin status			No. of women at:		Total no. of women examined
IgG	IgM	IgG avidity	≤20 weeks gestation	>20 weeks gestation	
-	-	ND	169	90	259
+	-	ND	202	106	308
+	+	Low	5	2	7
+	+	High	20	6	26

^a +, positive; -, negative; ND, not done; low, <35%; high, ≥35%.

TABLE 2. Screening data from seven women diagnosed as being at high risk for CMV intrauterine transmission^a

Case	Gestation at testing (wk)	Group	CMV IgG	CMV IgM (AxSYM)	CMV IgM (Eti-Cytok)	CMV IgG avidity (%)	PCR		CMV status at birth
							Urine	Blood	
1	7	A	+	+	+	22	-	+	Uninfected
2	12	A	+	+	+	33	+	-	Not tested
3	11	A	+	+	-	23	-	+	Uninfected
4	14	A	+	+	-	33	-	-	Uninfected
5	12	A	+	+	+	29	+	-	Uninfected
6	28	B	+	+	+	10	-	+	Infected
7	31	B	+	+	+	27	+	+	Infected

^a +, positive; -, negative.

sult had a natural miscarriage at 8 weeks gestation, and the products of conception were not obtained for testing (Table 2, case 2). Samples were obtained from all other infants within 3 days of birth, with neonatal blood and placental DNA tested by PCR and urine specimens tested by PCR and culture. All infants underwent hearing tests prior to their discharge from hospital. None of the infants in the control groups tested positive for CMV. Two cases of intrauterine transmission of CMV were detected from IgM-positive mothers who had low avidity IgG when first tested (Table 2, cases 6 and 7). Both mothers were from group B and had seroconverted during the second trimester of pregnancy. The two neonates were asymptomatic at birth, but were CMV positive in both blood and urine by CMV PCR and were positive for CMV urine culture. Both infants had normal hearing tests prior to discharge from the hospital and have been enrolled for regular follow-up hearing tests.

DISCUSSION

The base rate of CMV IgG seroprevalence in blood donors was shown to increase with age, from 34.9% at less than 20 years of age to 72.4% after the age of 50 years. These are the first published data of age-related CMV serostatus in an Australian population, and the results are similar to those from populations in Europe and the United States (21). Within the blood donor population significantly more women were infected with CMV than men, a finding in agreement with risk factors for other sexually transmitted viruses, such as human papillomavirus (29) and herpes simplex viruses (23). This increased risk is consistent with a demonstrated higher efficiency of male-to-female transmission (40).

Unlike the blood donor population, the rate of CMV infection in the pregnant-women cohort did not increase with age but instead was consistently high in women of less than 30 years of age (60 to 66%). Risk factors for CMV infection have been correlated with the socioeconomic status within a community (16, 17). The cohort of pregnant women was from the southeastern area of Sydney, which has the second highest socioeconomic status within the state of New South Wales (2a). The Australian data presented therefore differ from published data from the United States and Western Europe, in which women of childbearing age, of middle to upper class socioeconomic status, have a lower seroprevalence rate of CMV (21).

Serological screening was incorporated into already-established procedures, including first-trimester screening and GTT

screening for gestational diabetes in order to establish a diagnostic algorithm for CMV in pregnant women. Due to the advanced gestational age of many participants, the cohort was not as restricted as for previously published diagnostic algorithms (37, 43). A new algorithm was therefore designed (Fig. 1) based on initial detection with CMV IgG and IgM serological screening and the analysis of IgG avidity to determine the length of infection. Additional diagnostic tests, including CMV DNA detection in maternal blood and urine samples, were conducted to allow assessment of the serological diagnosis.

CMV IgM has been shown to peak during the first 1 to 3 months after primary infection in pregnant women and then persist at a low level for 18 to 39 weeks, with detection depending upon both the individual patient and the sensitivity of the IgM assay used (11, 43). After the initial onset of infection the rise in IgM titer may occur prior to the rise in IgG titer, making CMV IgG avidity testing reliant on the sensitivity of the CMV IgM test. To ensure that all CMV IgM-positive samples were detected and screened for IgG avidity, the AxSYM/IMx CMV IgM test was used, since it is one of the most sensitive commercial CMV IgM tests available (11, 32, 38). In agreement with published findings, the rate of IgM detection by the AxSYM/IMx was greater (5.7%) than the rate of the Eti-Cytok IgM (1.8%). Sensitive CMV IgM tests may have a high number of false-positive results (11, 38); however, all IgM results were verified by CMV IgG avidity testing, and the use of a sensitive CMV IgM test ensured that all individuals at risk of a primary infection were identified.

CMV IgG avidity has been shown to distinguish primary CMV infections from reactivated infections in pregnant women, and the maturation rate and duration of the antibody has been shown to correlate with patient viremia (4, 24, 33). Low-avidity IgG in pregnant women persists for approximately 17 weeks, with full maturation of the antibody occurring approximately 25 weeks after onset of symptoms (33). Screening in first trimester prior to 17 weeks gestation should therefore detect primary infections during this time. Combining CMV screening with GTT screening at 28 weeks gestation is a viable time to test for CMV infection in the second trimester since IgM from a primary infection will be detectable and IgG avidity can be determined and used in conjunction with retrospective screening of first trimester bleeds to determine the time of infection.

In immunocompetent persons, CMV detection in blood is thought to be indicative of a primary CMV infection (44). In the present study, CMV DNA was detected in blood samples

from three women with diagnosed primary CMV infection: one woman from group A (IgM positive with low-avidity IgG) and two women from group B who seroconverted. No correlation has been shown to exist between CMV viremia or CMV DNA detection in blood and intrauterine transmission (44); however, in the present study intrauterine transmission occurred in the two mothers from group B who were viremic at the time of testing.

CMV shedding in urine can occur in both primary and non-primary infections, and increasing numbers of women shed with advancing gestational age (19, 47). Only a small percentage (1.2%) of CMV seropositive women in the present study were found to be shedding CMV compared to published data (46). Urinary shedding has a poor correlation with intrauterine infection, although it has been identified as a risk factor (15, 42). In the present study one of the women from group B who seroconverted and was viremic at the time of testing was also shedding CMV in her urine and gave birth to a child with asymptomatic congenital CMV infection.

This prospective study provides a screening plan for women who are more advanced in gestation than allowed for by previous diagnostic algorithms. IgG avidity testing is less invasive than amniotic fluid testing and can be conducted during late gestation with no risk to the patient. Women infected with CMV during late gestation are more likely to transmit the virus to their unborn child than women who are infected early in gestation, with both cases of intrauterine transmission detected in the present study resulting from CMV infections in late gestation. Failure to detect seroconversions in late gestation women may result in failure to detect infected, asymptomatic neonates. Noninvasive serological testing with the improved algorithm will benefit such children, who are at a risk of complications, particularly sensorineural hearing loss, which can have a negative impact on the development of the child.

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